

**The use of microarray analysis to determine the gene expression
profiles of *Mycobacterium tuberculosis* in response to anti-bacterial
compounds**

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Abstract

The response of *Mycobacterium tuberculosis* to six antimicrobial agents was determined by microarray analysis in an attempt to define mechanisms of innate resistance in *M. tuberculosis*. The gene expression profiles of *M. tuberculosis* after treatment at the minimal inhibitory concentration (MIC) for 4 h with isoniazid, isoxyl, tetrahydrolipstatin, SRI#221, SR1#967 and SR1#9190 were compared to untreated *M. tuberculosis*. A common response to drug exposure was defined, and this expression profile overlapped with a number of other mycobacterial stress responses recently identified by microarray analysis. Compound-specific responses were also distinguished including a number of putative transcriptional regulators and translocation-related genes. These genes may contribute to the intrinsic resistance of *M. tuberculosis* to antimicrobial compounds. Further investigation into these mechanisms may elucidate novel pathways contributing to mycobacterial drug resistance and influence anti-mycobacterial drug development strategies.

Introduction

In 1993 the World Health Organisation declared tuberculosis (TB) a global emergency ¹. Ten years on, it has been estimated that the global incidence rate of tuberculosis is growing at approximately 0.4%/year ². Of the world's new tuberculosis cases approximately 3% were attributed to multidrug-resistant tuberculosis (MDR-TB) in 2000. Although multidrug-resistant tuberculosis may not be a problem globally, MDR-TB is at critical levels in many hot spots across the world ³. The emergence of MDR-TB, the deadly link between TB and HIV infection, the problems of treatment expense and patient compliance, and the requirement to eliminate persistent infection emphasises the need for new anti-mycobacterial compounds to be developed ⁴.

Mechanisms of drug resistance in *Mycobacterium tuberculosis* have been identified to all five first line anti-mycobacterial drugs – isoniazid (INH), rifampin, pyrazinamide, ethambutol, and streptomycin. *M. tuberculosis* multiple resistance in these instances is conferred by a series of chromosomal mutations ⁵. However, less is known about mechanisms of intrinsic/natural drug resistance in *M. tuberculosis* such as reduced cell wall permeability, efflux systems, or the expression of drug-inactivating enzymes. The poor action of many antibiotics, and the relative resistance of bacilli to drying, alkali and many chemical disinfectants has often been attributed to the low permeability of the unusual cell wall structure of mycobacteria ⁶. In addition to the hydrophobic barrier of the mycobacterial cell wall, several genes encoding putative drug efflux systems have been identified in mycobacteria. The

probable efflux protein *efpA* has been reported to be present in slow-growing pathogenic mycobacteria ⁷. The efflux pump LfrA has been demonstrated in *M. smegmatis* to confer low-level resistance to fluoroquinolones ⁸ and to contribute to ethidium bromide resistance ⁹, whereas the *M. tuberculosis* P55 multidrug efflux pump has been identified to confer aminoglycoside and tetracycline resistance ¹⁰. Indeed, the H37Rv *M. tuberculosis* genome sequencing project revealed the presence of up to 24 members of the major facilitator superfamily of transporters, and over 80 putative members of the ABC-transporter family ¹¹. Of the ABC-transporter family, 21 export systems were defined in *M. tuberculosis*, many of which are implicated in the export of drugs and which may contribute to the innate resistance of mycobacteria to broad spectrum antibiotics ¹². *M. tuberculosis* has also been demonstrated to express β -lactamases ¹³ and aminoglycoside acetyltransferases ¹⁴, which may reduce the effectiveness of β -lactams and aminoglycosides against *M. tuberculosis*. Further understanding of the mechanisms of intrinsic resistance to antibiotic compounds in *M. tuberculosis* may help to improve existing drug treatments and define new drug development strategies.

The advent of microarray technology has allowed the transcriptional profiles of bacteria to be examined in response to various stresses. The use of *M. tuberculosis* microarrays was first reported to describe the induction of *M. tuberculosis* genes in response to INH treatment ¹⁵. Genes were identified encoding proteins related to the mode of action of INH, such as *acpM* (coding for an acyl carrier protein), *kasA* and *kasB* (encoding β -ketoacyl synthases). Other genes most likely involved in the mycobacterial response to the toxicity

of the drug were also highlighted – *efpA* (coding for a putative efflux protein), and *aphC* (alkyl hydroperoxide reductase, involved in the oxidative stress response). Microarray analysis of gene expression has also recently been used to predict the common functional category of unknown anti-mycobacterial drugs as part of a pipeline of drug discovery ¹⁶.

We describe here the use of a gene-specific *M. tuberculosis* microarray to compare the transcriptional response of *M. tuberculosis* H37Rv to six compounds with anti-mycobacterial activity – (i) INH, a front line anti-tuberculosis drug targeting mycolic acid synthesis ¹⁷; (ii) Isoxyl, a drug used in the past to treat tuberculosis, targeting a delta-9 oleic acid desaturase and mycolic acid synthesis ^{18/19}; (iii) Tetrahydrolipstatin (THL), a lipase inhibitor used in the treatment of obesity ²⁰; and three compounds from The Southern Research Institute (Birmingham, Alabama, USA), exhibiting potent anti-mycobacterial properties (iv) SRI#221; (v) SRI#967; and (vi) SRI#9190. The comparison of these six distinct transcriptional profiles defines a common *M. tuberculosis* response to these anti-mycobacterial compounds, and describes drug-specific changes which may reflect the mode of action of each drug. This investigation also distinguishes genes of unknown function that may contribute to the intrinsic resistance of *M. tuberculosis* to antimicrobial agents.

Materials and Methods

Growth Conditions and RNA Extraction

Mycobacterium tuberculosis strain H37Rv was grown at 37°C in Dubos liquid medium, supplemented with bacto Dubos medium albumin (Becton Dickinson). Mid-log phase mycobacterial cultures were concentrated to 1/20th volume of liquid medium and incubated overnight to recover. Antimicrobial compounds were added at approximately x1 MIC (determined using the Microplate Alamar Blue Assay, MABA ²¹, at The Southern Research Institute); the structures and MICs of the compounds used are detailed in Fig. 1. The drug-treated, together with untreated control mycobacterial cultures, were incubated at 37°C for 4 hours. *M. tuberculosis* RNA was extracted using the GTC/TRIzol[®] method developed by Mangan *et al.*, 1997 ²². The RNA samples were DNaseI treated and cleaned up on RNeasy[®] Mini-Columns (Qiagen).

Microarray Hybridisation and Normalisation Strategies

cDNA derived from three separate RNA extractions for each of the compounds tested and from untreated control samples were hybridised to a gene-specific PCR product H37Rv *M. tuberculosis* microarray, the design and generation of which is described in Stewart *et al.*, 2002 ²³. Details of the *M. tuberculosis* microarray used in this investigation can be found at <http://bugs.sghms.ac.uk/>. Two colour competitive hybridisations were performed as previously described Stewart *et al.*, 2002 ²³ hybridising the mycobacterial RNA-derived cDNA against *M. tuberculosis* genomic DNA. The hybridised slides were scanned sequentially at 532 nm and 635 nm corresponding to Cy3 and Cy5 excitation maxima using the 4.28[™] Array

Scanner (Affymetrix). Comparative spot intensities from the images were calculated using Imagene 4.0 (BioDiscovery), and imported into GeneSpring 4.2 (Silicon Genetics) for further analysis. The array data was normalised to the 50th percentile, and values of less than zero were adjusted to zero. Repeat hybridisations using the same cDNA samples (between 3-7 replicates for each condition) were replicated together. The experiments were then normalised to the untreated control sample using a per gene normalisation strategy.

Microarray Data Analysis

Two measures of significance were applied to the normalised data set to identify differentially regulated genes (i) a minimum p value of 0.05 incorporating the cross-gene error model (GeneSpring) was set to discriminate genes significantly deviating from the 1:1 ratio (treated:untreated) which were then subjected to Benjamini and Hochberg correction to take into account multiple experiment testing and (ii) a one-way ANOVA (GeneSpring).

A technique of single spot replacement, SSR, (J. Bacon, personal communication) was also used to enhance the original data set. The un-normalised cDNA:genomic DNA ratios for each replicate under each condition were imported into Microsoft Excel. For each element on the microarray the individual ratio furthest from the median of the replicates was replaced with the mean of the remaining ratios. In this way the effect of extreme values was minimised from the data set. This SSR data set was then normalised as previously described, and subjected to two measures of significance: (i) the statistical group comparison (ANOVA); and (ii) the statistical package SAM

(Significance Analysis of Microarrays, version 1.15 ²⁴) was used to identify genes differently expressed in the normalised data sets. A minimum fold change of 1.5 between control and drug-treated data sets, and a false discovery rate (FDR) of less than one (of the median) was used as a measure of significance.

The hypergeometric distribution was used to determine if particular functional categories of genes were enriched in response to each drug treatment. The hypergeometric p values were calculated as described by Boldrick *et al.*, 2002 ²⁵; where $N = 3924$ the total number of genes in the population, A = the number of genes within each functional classification, x = the number of genes identified as up-regulated in response to each drug, and n = the total number of genes up-regulated after treatment by each anti-microbial compound.

Results

The transcriptional response of *M. tuberculosis* to each of the six antimicrobial agents was defined as the subset of genes identified as significantly differentially expressed in two or more statistical tests (described in the Methods). Using this analysis strategy, 155 genes were demonstrated to be up-regulated by INH treatment (32 down-regulated); Isoxyl (231 up, 21 down); Orlistat (208 up, 24 down); SRI#221 (182 up, 25 down); SRI#967 (116 up, 30 down); and SRI#9190 (124 up, 22 down). The fold changes and predicted function of these genes are described as supplementary information Table S1.

Dissecting the transcriptional response of *M. tuberculosis* to the six drug compounds by functional classification (as described by Cole *et al.*, 1998 ¹¹) revealed that the genes induced by drug treatment broadly represent most of the range of pathways that are present in *M. tuberculosis*. The hypergeometric distribution ²⁵ was used to determine whether the enrichment of genes within a particular functional category in response to each drug treatment was significant (p value < 0.05). Table 1 shows that the number of genes within the functional categories of energy metabolism and chaperones/heat shock were significantly enhanced after treatment with each of 3 or more anti-microbial agents. The functional category of lipid metabolism was significantly enriched in response to INH and isoxyl treatment, as was the category of polyketide synthesis after treatment with SRI#967. Additionally, the proportion of genes involved in the metabolism of the cell envelope was significantly increased after treatment with SRI#221 and SRI#967 (Table 1).

Common response to anti-mycobacterial agents

By comparing the similarities between the *M. tuberculosis* drug-induced expression profiles, a common response to anti-mycobacterial agents could be defined. A subset of 80 genes were identified which were significantly up-regulated after treatment with 3 or more anti-microbial compounds (of a maximum 6). These genes are listed in Table 2. Many of these common genes induced by exposure to anti-microbial compounds were involved in the mycobacterial stress response. Genes associated with DNA repair such as *end* (coding for a probable endonuclease) and *recA* (encoding recombinase A²⁶) were up-regulated; together with *Rv3049c* (a probable monooxygenase) and *aphC* (alkyl hydroperoxide reductase) expressed in response to oxidative stress²⁷. Also over-expressed after drug treatment (>3 drugs) were *gltA1* (a probable citrate synthase) and *icl* (isocitrate lyase) similar to changes in metabolism seen under stress conditions²⁸. RNA polymerase sigma factors A and B were also induced, together with serine/threonine protein kinases B and G. *sigB* has been implicated in the *M. tuberculosis* response to a number of stress conditions²⁹. The product of *pknG* is predicted to be a soluble protein (the transcription of which may be controlled by the redox status of the cell) which may be involved in glutamine uptake and which may be up-regulated under nitrogen-limiting conditions³⁰. Additionally the putative nitrate/nitrite transporter *narK2*, the nitroreductase *acg* and the nitrate reductase *narH* were also identified to be induced. Indeed, five genes demonstrated to be part of the ACG (acr-coregulated gene) family were found to be up-regulated after exposure to anti-mycobacterial compounds – *Rv1733c*, *narK2*, *Rv1738*, *Rv2005c*, and *acg*³¹.

Many of the 'common' genes induced by drug treatment have been identified as part of the mycobacterial response to other stresses such as low oxygen ³², heat shock ²³, acid shock ³³, detergent stress ³⁴, nitric oxide treatment ³⁵, phagocytosis ³⁶ or nutrient/carbon starvation ³⁷/Hampshire *et al.*, this issue. Those genes, which have also been previously identified to be up-regulated in response to these other stresses are marked in Table 2. Of the remaining genes induced by 3 or more drugs, five are annotated as efflux proteins or transporters – *nark2* (a possible nitrate/nitrite transporter), *Rv1747* (a probable ABC transporter), *ctpF* (a putative metal cation transporter), *efpA* (an efflux protein), and *ctpC* (a probable metal cation transporter). A second subset of six genes belonging to the ESAT-6 family of proteins was also identified as up-regulated after 4 h drug exposure (*Rv1037c*, *Rv1197-Rv1198*, *Rv1793*, *Rv2346c*, and *Rv3874*). The ESAT-6 gene clusters in *M. tuberculosis* have been associated with the generation and transportation of T-cell antigens lacking detectable secretion signals ³⁸. These genes linked to the transportation of unknown moieties may be directly involved in the mycobacterial response to drug compounds which contributes to the intrinsic resistance of mycobacteria to antimicrobial agents.

Drug-specific expression responses

The expression profiles of *M. tuberculosis* treated with each of the six antimicrobial compounds were compared, by generating a similarity matrix detailing the number of overlapping genes between two drug treatments as a

proportion of the possible maximum (Fig. 2). The mycobacterial response to INH and isoxyl exposure was most similar as may be expected as both drugs target aspects of fatty acid and mycolic acid biosynthesis ^{17/18/19}. The expression profiles of *M. tuberculosis* treated with the compounds SRI#967 and SRI#9190 were also similar. Aspects of the mycobacterial response to individual drugs focusing on the possible action of the compounds is briefly presented below.

INH and Isoxyl

Both INH and isoxyl inhibit fatty acid and mycolic acid biosynthesis in *M. tuberculosis* ^{17/18/19}. INH has been demonstrated to target the enoyl-AcpM reductase *InhA*, a component of the fatty acid synthase – II (FAS-II) ³⁹. Isoxyl treatment inhibits mycolic acid and shorter-chain fatty acid synthesis leading to the hypothesis that isoxyl may act on other components of FAS-II ¹⁸. Interestingly, it has also recently been shown to target a delta-9 desaturase in mycobacteria ¹⁹. Genes coding for enzymes involved in FAS-II were up-regulated after exposure to both drugs: *fabD* (coding for a malonyl-CoA::acyl carrier protein (ACP) transferase), *acpM* (an acyl carrier protein), *kasA* and *kasB* (both β -ketoacyl ACP synthases). These have been previously identified to be induced by INH, ethionamide and thiolactomycin treatment ^{15/16}. Interestingly amongst other fatty acid biosynthetic genes induced by isoxyl treatment alone was *mabA*, a gene coding for a β -ketoacyl ACP reductase, which also belongs to the FAS-II system. The induction of *mabA* (which is transcriptionally linked to *inhA* in *M. tuberculosis*), after exposure to isoxyl, but not INH, may reflect differences in the mode of action of these two

compounds. Further experiments such as the overexpression of *mabA* during isoxyl exposure may help to elucidate the primary target of isoxyl ⁴⁰.

Tetrahydrolipstatin

Tetrahydrolipstatin (THL) is a reversible inhibitor of lipases used in the treatment of obesity (Xenical®, Roche). A number of *M. tuberculosis* putative lipases were up-regulated in response to THL treatment – *Rv1683*, *lipD* and *lipV* (although this was not significant by hypergeometric testing). Of the remaining induced genes, 4 encoding putative transporters (*ctpI*, *sugA*, *Rv3253c*, and *Rv3781*) and 6 coding for probable transcriptional regulators were identified (*Rv0043c*, *Rv0823c*, *sigE*, *Rv3167c*, *Rv3687c* and *Rv3855*). Also up-regulated on exposure to THL were 4 genes located in a gene cluster *Rv0676c-Rv0679c*. *Rv0676c* (*mmpL5*) belongs to a family of conserved large membrane proteins, *Rv0677c* (*mmpS5*) is part of a related small membrane protein family (which appears to overlap stop and start codons with *mmpL5*), the function of *Rv0678* is unknown, and *Rv0679c* codes for a threonine-rich protein of undetermined function. The functional significance of this cluster of genes in the *M. tuberculosis* response to THL treatment cannot be elucidated by microarray analysis alone. However, further experimentation into this cluster or other genes of interest may define novel mechanisms of drug resistance in *M. tuberculosis*.

SRI#221

Fig. 1 shows that SRI#221 is a potent antitubercular compound with a low MIC value, however the primary mode of action of this anti-microbial

compound is unknown. Treatment of *M. tuberculosis* with SRI#221 induced two clusters of genes involved in complex lipid biosynthesis. The first cluster containing *tesA* (a probable thioesterase), *drrB* (an ABC-type transporter), *papA5* (a polyketide synthase associated protein), and *fadD28* (a fatty acid-CoA ligase), is involved in the biosynthesis and translocation of the multi-methyl branched mycocerosic acids in the generation of phthiocerol dimycocerosates ⁴¹. The second gene cluster includes *Rv3822* (of unknown function), *mmpL8* (a conserved large membrane protein), *papA1* (a polyketide synthase associated protein) and *fadD23* (a probable fatty acid-CoA ligase). These genes cluster around the polyketide synthase *pks2*, responsible for the biosynthesis of the multi-methyl branched phthioceranic acids present in the sulpholipid complex lipids ⁴². The induction of these gene clusters may be part of a compensatory network to minimise the anti-mycobacterial effects of SRI#221, may reflect the broad nature of SRI#221 action, or highlight the primary mode of SRI#221 action to be within shared basic lipid biosynthetic pathways.

SRI#967 and SRI#9190

The mode of action of the anti-mycobacterial compounds SRI#967 and SRI#9190 are yet to be determined. The *M. tuberculosis* response to SRI#967 exposure includes the up-regulation of *Rv0076c* and *Rv0077c*. *Rv0076c* encodes a probable membrane protein, whereas *Rv0077c* codes for a possible oxidoreductase. Similarly, *Rv0135c* (a putative transcriptional regulator) and *Rv0136* (belonging to the cytochrome P450 group of monooxygenases) were induced by SRI#9190 treatment alone. The induction

of these two distinct clusters (on exposure to different anti-mycobacterial compounds) may be part of a similar oxidative stress response. The identification that these genes may play a role in the intrinsic resistance of *M. tuberculosis* to anti-microbial agents using microarray analysis enables more specific experimental strategies to be employed.

Of particular interest was a cluster of 4 genes which were significantly induced on exposure to both SRI#967 and SRI#9190. *Rv3159c* (encoding PPE53, a member of the PPE family), *Rv3160c* (a putative transcriptional regulator), *Rv3161c* (a probable dioxygenase) and *Rv3162c* (a possible integral membrane protein) were up-regulated on exposure to these compounds alone (none of these genes were induced by the other anti-microbial agents tested). The probable dioxygenase *Rv3161c* is most similar to ring hydroxylating dioxygenases, so it likely that this enzyme is involved in the degradation of benzene ring structures (which SRI#967 and SRI#9190 both contain, Fig. 1). *Rv3160c* and *Rv3161c* have recently been identified to be induced by triclosan treatment ¹⁶. Triclosan (2,4,4'-trichloro-2-hydroxydiphenyl ether) contains two chlorinated benzene rings. This cluster of genes may therefore be induced on exposure to compounds containing halogenated benzene rings, this would explain the up-regulation of the cluster in response to SRI#967 and SRI#9190, but not to the benzene ring structures in isoxyl and SRI#221. This gene cluster may be induced as part of a response to render halogenated benzene compounds benign, so contributing to the natural resistance of *M. tuberculosis* to a range of anti-microbial agents.

Discussion

The response of *M. tuberculosis* to six antimicrobial compounds was determined by microarray analysis. The microarray expression data set was analysed using several statistical methods, the use of multiple statistical methods added extra depth to the interpretation of the data sets. Additionally, a single spot replacement strategy was used alongside the original data set to allow the maximum amount of information to be extracted from the data sets without significantly shifting the expression patterns.

Using this microarray analysis strategy, elements of a *M. tuberculosis* common stress response and specific drug-induced changes were identified to six antimicrobial compounds. The up-regulation of genes specific to each compound may reflect the mode of action of the drug or define innate resistance mechanisms in *M. tuberculosis*. This investigation has defined an initial subset of genes which may be important in the innate resistance of *M. tuberculosis* to antimicrobial agents. Microarray profiling of *M. tuberculosis* gene expression after exposure to drugs enables compounds of unknown mechanism of action to be classified into similarity groups, but in this study has not been helpful to elucidate the site of or mechanism of action. This would require complementary genetic and biochemical studies informed by microarray profiling.

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Figure 1 – The structures of the antimicrobial compounds used in this investigation. The source and the approximate MIC of the compounds against *M. tuberculosis* are also detailed. MICs were determined using the Microplate Alamar Blue Assay (MABA) ²¹ at The Southern Research Institute (Birmingham, Alabama, USA).

Figure 2 – A similarity matrix comparing the genes up-regulated in response to the 6 anti-microbial compounds tested (isoniazid, INH; isoxyl, ISO; tetrahydrolipstatin, THL; SRI#221/967/9190). Numbers in the top half of the figure represent the number of common genes up-regulated in response to two compounds. The maximum number of genes identified as significantly up-regulated (in more than 2 statistical tests) after treatment with each drug are displayed in the shaded cells. The bottom half of the matrix describes the number of genes common to two drug responses as a proportion of the possible maximum, calculated as $1 - (\text{common genes} / \text{maximum possible genes})$. The smaller this proportion is the greater the extent of the overlap between expression responses.

Table 1 – The *M. tuberculosis* response to 6 anti-microbial compounds examined by functional category, as defined by Cole *et al.*, 1998 ¹¹. Only genes identified as significantly over-expressed in two or more of the statistical tests described in response to isoniazid (INH), isoxyl (ISO), tetrahydrolipstatin (THL), SRI#221/967/9190 are detailed in these tables. These gene lists are described in Supplementary Table S1. The hypergeometric probabilities ²⁵ of the enrichment of particular functional

categories of genes in response to each drug treatment are indicated if ^a p value < 0.05, ^b < 0.01, ^c < 0.001.

Table 2 – The common response of *M. tuberculosis* to 6 anti-microbial agents, detailing the genes identified to be up-regulated in response to 3 or more of the anti-microbial compounds tested (maximum 6). Numbers in the columns (INH isoniazid, ISO isoxyl, THL tetrahydrolipstatin, SRI# 221/967/9190) indicate the number of statistical tests in which each gene was found to be significantly induced (minimum of 2, maximum 4). The left column labelled N details the number of drugs in which each gene was significantly differentially expressed. Dots present in the columns A-J indicate that the gene has been previously identified to be up-regulated in response to various other stresses; A INH treatment ¹⁵, B INH and TLM treatment ¹⁶, C low oxygen ³², D nutrient starvation ³⁷, E nitric oxide treatment ³⁵, F phagocytosis ³⁶, G carbon starvation (Hampshire *et al.*, this issue), H detergent stress ³⁴, I heat shock ²³, J acid shock ³³. This table is ordered by Rv number.

Supplementary Data Table S1 – The differentially regulated genes in response to 6 anti-microbial compounds. These tables describe the genes identified as significantly differentially expressed in the *M. tuberculosis* responses to each of the six anti-microbial compounds tested. Each table consists of the gene name (and unique gene identifier), Rv number, a brief description of the proposed function of the gene and the fold expression ratios determined using each of the four statistical tests described. The number of times each gene has been identified as significantly differentially expressed is

detailed in the column labelled N. Cells in this column coloured purple indicate the presence of consecutive Rv numbers. Only genes identified by two or more statistical tests have been included in these tables. These tables are ordered by Rv number.

Figure 1

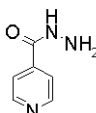
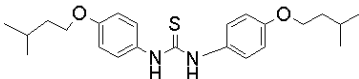
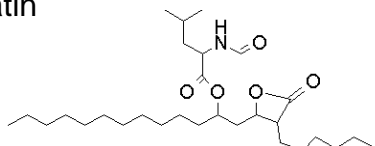
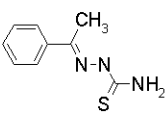
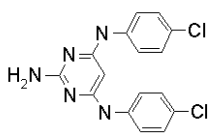
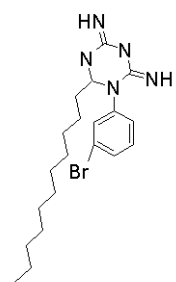
Structure of Drug Compound Tested	Approximate MIC	Source
Isoniazid 	0.2 µg/mL	Sigma I-3377
Isoxyl 	2 µg/mL	NIMR, UK
Tetrahydrolipstatin 	50 µg/mL	Hoffmann-La Roche
SRI#221 	0.4 µg/mL	SRI, USA
SRI#967 	6.25 µg/mL	SRI, USA
SRI#9190 	6.25 µg/mL	SRI, USA

Table 1

Functional Classification (as defined by Cole <i>et al.</i> , 1998 ¹¹)	Genes identified as up-regulated					
	INH	ISO	THL	221	967	9190
I. Small-molecule Metabolism						
A. Degradation (163)	8	8	8	5	8	7
B. Energy Metabolism (292)	10	32 ^c	18	18 ^a	9	14 ^a
C. Central Intermediary Metabolism (45)	3	3	1	4		1
D. Amino Acid Biosynthesis (95)	4	7	1	2	2	2
E. Polyamine Synthesis (1)						
F. Purines, Pyrimidines, Nucleosides and Nucleotides (60)	2	2		5		
G. Biosynthesis of Cofactors, Prosthetic Groups and Carriers (117)	5	2	4	7	2	3
H. Lipid Biosynthesis (65)	7 ^b	11 ^c	4	5	3	3
I. Polyketide and Non-Ribosomal Peptide Synthesis (41)	4	3	2	3	4 ^a	2
J. Broad Regulatory Functions (187)	4	5	7	9	8	5
II. Macromolecule Metabolism						
A. Synthesis and Modification of Macromolecules (215)	10	17 ^a	17 ^a	12	8	10
B. Degradation of Macromolecules (87)	3	7	5	5	1	2
C. Cell Envelope (360)	16	23	18	26 ^b	20 ^b	9
III. Cell Processes						
A. Transport/Binding Proteins (123)	7	7	9	4	1	3
B. Chaperones/Heat Shock (16)	3 ^a	6 ^c	3 ^a	1		3 ^a
C. Cell Division (19)		2	3	1		
D. Protein and Peptide Secretion (14)		2	1	1		1
E. Adaptations and Atypical Conditions (12)					1	
F. Detoxification (22)		1	1	3	1	1
IV. Other						
A. Virulence (38)			1	1	1	
B. IS Elements, Repeated Sequences and Phage (135)	9 ^a	1	11 ^a	1	3	
C. PE and PPE Families (167)	10	6	9	2		4
D. Antibiotic Production and Resistance (14)			1	1		1
E. Bacteriocin-like Proteins (3)				1		
F. Cytochrome P450 Enzymes (22)	1	1	2	3	1	1
G. Coenzyme F420-dependent Enzymes (3)		1				
H. Miscellaneous Transferases (61)	1	3	4	2	3	2
I. Miscellaneous Phosphatases, Lyases and Hydrolases (18)						1
J. Cyclases (6)			1	1		
K. Chelataes (2)						
V. Conserved Hypotheticals (915)	36	61	40	26	29	30
VI. Unknowns (606)	12	20	37	33	11	19
Total Genes (3924)						
Total up-regulated genes after drug treatment	155	231	208	182	116	124
Percentage of unknown function (39% of total genes unknown)	31	35	37	32	34	40

Figure 2

	INH	ISO	THL	#221	#967	#9190
INH	155	67	27	19	24	30
ISO	0.568	231	44	36	39	44
THL	0.826	0.788	208	32	29	25
#221	0.877	0.802	0.824	182	36	30
#967	0.793	0.664	0.750	0.690	116	44
#9190	0.758	0.645	0.798	0.758	0.621	124

Table 2

N	Gene Name	Rv No	INH	ISO	THL	221	967	9190	Putative Function	A	B	C	D	E	F	G	H	I	J
3	<i>pknB</i>	<i>Rv0014c</i>				2	3	3	Serine/threonine protein kinase B								•		
4	<i>rpsF</i>	<i>Rv0053</i>		2		2	3	3	Ribosomal protein S6			•							
5	<i>icd2</i>	<i>Rv0066c</i>		2	2	3	3	3	Isocitrate dehydrogenase			•					•		
3	<i>fbpC2</i>	<i>Rv0129c</i>		4	2			3	Antigen 85c, mycolyl transferase C	•			•						
4	<i>pntAB</i>	<i>Rv0156</i>	2	3	2	3			Probable NAD(P) transhydrogenase										
4	<i>bglS</i>	<i>Rv0186</i>	2	2			2	3	Probable beta-glucosidase								•		
4	<i>Rv0247c</i>	<i>Rv0247c</i>		2	3		3	2	Probable succinate dehydrogenase										
3	<i>fadD2</i>	<i>Rv0270</i>	4		2			2	Probable long-chain fatty acid CoA ligase										
3	<i>Rv0349</i>	<i>Rv0349</i>	2		2	3			Unknown										
3	<i>pknG</i>	<i>Rv0410c</i>		2	2	2			Serine/threonine protein kinase G			•							
3	<i>Rv0412c</i>	<i>Rv0412c</i>	2	2		2			Possible conserved membrane protein			•				•			
3	<i>Rv0446c</i>	<i>Rv0446c</i>		3		2	2		Possible conserved membrane protein						•				
3	<i>icl</i>	<i>Rv0467</i>	4	2				2	Isocitrate lyase						•	•	•		•
3	<i>mmpS2</i>	<i>Rv0506</i>				2	2	3	Unknown, probable membrane protein										
3	<i>end</i>	<i>Rv0670</i>	2	2				2	Probable endonuclease IV			•	•			•			
4	<i>rpsS</i>	<i>Rv0705</i>		2	2		3	4	30s ribosomal protein s19										
4	<i>rpmC</i>	<i>Rv0709</i>		3	2		2	4	50s ribosomal protein L29										
3	<i>rplR</i>	<i>Rv0720</i>	4	2				2	50s ribosomal protein L18										
3	<i>Rv0851c</i>	<i>Rv0851c</i>				2	2	2	Probable dehydrogenase/reductase						•				
4	<i>fadE10</i>	<i>Rv0873</i>	4	4	2		2		Probable acyl-CoA dehydrogenase							•	•		
3	<i>Rv0910</i>	<i>Rv0910</i>		2	2	2			Unknown										
3	<i>sucC</i>	<i>Rv0951</i>	3	3	2				Probable succinyl-CoA synthetase		•								
4	<i>esxI</i>	<i>Rv1037c</i>	4	3			3	3	Putative ESAT-6 like protein								•		
5	<i>Rv1109c</i>	<i>Rv1109c</i>	2	3		3	3	2	Unknown							•			
3	<i>gltA1</i>	<i>Rv1131</i>	4	2	2				Probable citrate synthase						•	•	•		•
4	<i>narH</i>	<i>Rv1162</i>	2	2		2		2	Probable respiratory nitrate reductase			•					•		
3	<i>papA3</i>	<i>Rv1182</i>			3		3	3	Polyketide associated protein				•					•	
3	<i>Rv1184c</i>	<i>Rv1184c</i>		2	3		3		Unknown, possible exported protein						•				
4	<i>esxK</i>	<i>Rv1197</i>	3	2			3	3	Putative ESAT-6 like protein										

4	<i>esxL</i>	<i>Rv1198</i>	4	4			2	4	Putative ESAT-6 like protein										
3	<i>atpG</i>	<i>Rv1309</i>		2	2	2			ATP synthase gamma chain										
3	<i>PPE19</i>	<i>Rv1361c</i>	4	4			2		PPE family protein										
4	<i>appC</i>	<i>Rv1623c</i>	2	2		3		3	Probable cytochrome D ubiquinol oxidase										
4	<i>Rv1683</i>	<i>Rv1683</i>	2		2		2	2	Possible long-chain acyl-CoA synthase		•								
4	<i>Rv1733c</i>	<i>Rv1733c</i>	4	4			3	4	Probable conserved transmembrane protein		•		•	•					
3	<i>narK2</i>	<i>Rv1737c</i>	4	3	2				Possible nitrate/nitrite transporter		•		•	•					
4	<i>Rv1738</i>	<i>Rv1738</i>		4		2	2	3	Unknown		•		•	•				•	
3	<i>Rv1747</i>	<i>Rv1747</i>		2	2		2		Probable membrane transport protein										
4	<i>esxN</i>	<i>Rv1793</i>	3	3			3	3	Putative ESAT-6-like protein										
4	<i>Rv1813c</i>	<i>Rv1813c</i>	2	4	2			2	Unknown			•		•	•				
4	<i>Rv1987</i>	<i>Rv1987</i>	3		4	3	3		Probable chitinase		•		•						
3	<i>ctpF</i>	<i>Rv1997</i>		2	2			2	Probable metal cation transporter			•		•	•				
4	<i>Rv1998c</i>	<i>Rv1998c</i>		2	2		2	2	Unknown			•		•					
3	<i>Rv2005c</i>	<i>Rv2005c</i>	4	4	2				Unknown			•		•	•			•	
3	<i>fdxA</i>	<i>Rv2007c</i>	2	4				2	Probable ferredoxin		•	•		•	•			•	•
6	<i>acg</i>	<i>Rv2032</i>	3	4	2	3	3	4	Unknown, possible nitroreductase			•		•	•				
4	<i>Rv2091c</i>	<i>Rv2091c</i>		3		2	3	4	Unknown, probable membrane protein				•			•			
3	<i>Rv2147c</i>	<i>Rv2147c</i>		3	2			3	Unknown protein										
3	<i>Rv2185c</i>	<i>Rv2185c</i>		2		2		2	Unknown (TB16.3)				•					•	
5	<i>cbhK</i>	<i>Rv2202c</i>	3	3	2		2	3	Probable carbohydrate kinase									•	
4	<i>fabD</i>	<i>Rv2243</i>	2	4	2	3			Malonyl CoA-acyl carrier transacylase	•	•	•							
5	<i>acpM</i>	<i>Rv2244</i>	4	4		4	3	3	Meromycolate extension acyl carrier protein	•	•	•							
4	<i>kasA</i>	<i>Rv2245</i>	2	2		2	2		Beta-ketoacyl-ACP synthase	•	•		•						
3	<i>htpG</i>	<i>Rv2299c</i>		4	3	3			Probable heat shock protein									•	
4	<i>esxO</i>	<i>Rv2346c</i>	3	3			2	4	Putative ESAT-6 like protein										
3	<i>Rv2405</i>	<i>Rv2405</i>	2	2		3			Unknown				•						
3	<i>ahpC</i>	<i>Rv2428</i>			3	4	3		Alkyl hydroperoxide reductase C	•	•	•			•			•	
4	<i>pepD</i>	<i>Rv2467</i>	3	4	2		3		Probable aminopeptidase (<i>pepM</i>)										
5	<i>Rv2626c</i>	<i>Rv2626c</i>	3		2	2	2	2	Unknown			•		•	•				
3	<i>Rv2627c</i>	<i>Rv2627c</i>		4		2	2		Unknown			•		•	•				
3	<i>Rv2629</i>	<i>Rv2629</i>			2	2		2	Unknown			•		•	•				
3	<i>sigA</i>	<i>Rv2703</i>				3	2	3	RNA polymerase sigma factor A									•	

4	<i>sigB</i>	<i>Rv2710</i>	3		2	2		4	RNA polymerase sigma factor B				•		•	•	•	•	
3	<i>recA</i>	<i>Rv2737c</i>		2	2			3	Recombinase A protein										
6	<i>35kd_ag</i>	<i>Rv2744c</i>	2	2	2	2	2	3	35-kd alanine rich antigen						•	•	•	•	
4	<i>thyX</i>	<i>Rv2754c</i>		2		2	2	3	Probable thymidylate synthase										
3	<i>Rv2818c</i>	<i>Rv2818c</i>				3	4	3	Unknown										
3	<i>efpA</i>	<i>Rv2846c</i>	4	4	2				Putative efflux protein	•	•								
3	<i>Rv2959c</i>	<i>Rv2959c</i>		2	2	3			Possible methyltransferase										
5	<i>Rv3049c</i>	<i>Rv3049c</i>		2	2	2	2	2	Probable monooxygenase										
3	<i>PPE51</i>	<i>Rv3136</i>	4	2				2	PPE family protein			•							
3	<i>rubB</i>	<i>Rv3250c</i>	3	3				2	Probable rubredoxin					•					
3	<i>ctpC</i>	<i>Rv3270</i>		2	2			2	Probable metal cation transporter			•			•				
3	<i>PE_PGRS52</i>	<i>Rv3388</i>	2	2			2		PE_PGRS family protein										
3	<i>PPE60</i>	<i>Rv3478</i>	4	4	2				PPE family protein										
3	<i>Rv3592</i>	<i>Rv3592</i>	3	3			2		Unknown (TB11.2)						•				
4	<i>panD</i>	<i>Rv3601c</i>	2			2	2	4	Aspartate1-decarboxylase										
3	<i>mmpL8</i>	<i>Rv3823c</i>				2	2	2	Conserved large membrane protein										
3	<i>papA1</i>	<i>Rv3824c</i>				2	2	2	Polyketide associated protein			•				•			
5	<i>esxB</i>	<i>Rv3874</i>		2	2	2	2	2	10 KDa culture filtrate antigen (<i>cfp10</i>)										